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Research Article

Hydrogen-Rich Saline Postconditioning and Mitochondrial Function in Rats Undergoing Ischemia-Reperfusion Injury

Guoming Zhang[1], LichengDing[1], Guangfeng Sun[1], Longxing Cao[1], Bin Wang[1], Gary Tse[2] and Yuanyuan Sun[1]*

[1]Department of Cardiology, Xiamen Cardiovascular Hospital, Xiamen University, China

[2]Kent and Medway Medical School, China

Abstract

Mechanical postconditioning can alleviate myocardial I/R injury. The present study aimed to investigate whether pharmacological postconditioning using hydrogen-rich saline may alleviate mitochondrial dysfunction by preserving complex III activity following I/R injury in rats.

In our study, 96 male and female Sprague-Dawley rats were randomly divided into four groups: sham, I/R, I/R with hydrogen-rich saline, and I/R with hydrogen-rich saline and antimycin A. The animals were sacrificed at 5 min, 6 or 24 h, and the levels of Reactive Oxygen Species (ROS), complex III activity, mitochondrial function, cytochrome b and c, serum markers, apoptosis, and infarct size were determined.

Following I/R, the expression levels of cytochrome b, complex III activity, and mitochondrial transmembrane potential in the hydrogen-rich saline group were significantly higher ($P<0.05$), whereas the expression levels of cytochrome c, myocardial serum markers, apoptotic index, and infarction area were significantly lower ($P<0.05$) than those in the I/R group. Rats in the hydrogen-rich saline and antimycin A-treated groups exhibited significant improvement in the aforementioned variables compared with those in the I/R group, but only moderate improvement compared with the hydrogen-rich saline group ($P<0.05$).

In conclusion, hydrogen-rich saline postconditioning increased complex III activity and mitochondrial transmembrane potential and alleviated myocardial apoptosis in rats following I/R injury.

Keywords: Reperfusion injury; Mitochondria; Complex III; Pharmacological postconditioning; Hydrogen saline

Abbreviations

AMI: Acute Myocardial Infarction; STEMI: ST-Elevation Myocardial Infarction; I/R: Ischemia-Reperfusion; ROS: Reactive Oxygen Species; MDA: Malondialdehyde; ·OH: HydroxylRadical; SOD: Superoxide Dismutase; Cyt-b: Cytochrome b; Cyt-c: Cytochrome c; mPTP: mitochondrial Permeability Transition Pore; TnT: Troponin T; CK-MB: MB Isoenzyme of Creatine Kinase; AAR: Myocardium at Risk; AN: Area of Necrosis; LV: Left Ventricular; MAP: Mean Arterial Pressure; PPC: Pharmacological Postconditioning; I-PostC: Ischemic Postconditioning

Introduction

In recent years, acute myocardial infarction has become one of the leading causes of death globally. Each year in the United States alone, more than one million patients will be hospitalized for Acute Myocardial Infarction (AMI) or coronary heart death. The rate of AMI rises sharply, especially in developing countries such as China and India [1]. The overall number of deaths has declined steadily over the past 30 years, but it has stabilized over the past decade, and the short-term mortality rate of patients with ST-Elevation Myocardial Infarction (STEMI) ranges from 5% to 6% during the initial hospitalization and from 7% to 18% at 1 year [2]. Despite the development of effective reperfusion therapy that aims to open up the occluded coronary vessels, adverse events such as ischemia-reperfusion (I/R) injury still occur [3]. Previous studies from our group and other groups have investigated the protective effects of postconditioning to reduce reperfusion-related injury [4-9]. Postconditioning can be achieved mechanically through repeated expansion at the lesion site during percutaneous coronary intervention, but its use is limited by complications such as plaque rupture, thrombosis, embolism, and coronary artery dissection. Therefore, pharmaceutical postconditioning has been explored for its potential benefits [10-12].

Hydrogen selectively antagonizes Reactive Oxygen Species (ROS) such as hydroxyl free radicals and peroxynitrite anions [13,14]. Mitochondria are the main organelles involved in the formation of oxygen free radicals, and complex III is the major site of ROS formation. Our previous study demonstrated that hydrogen saline significantly reduced the levels of oxygen free radicals in rat myocardial cells as well as the area of myocardial infarction [6]. In the past, the relationship between hydrogen saline and mitochondrial respiratory chain complex III has been rarely studied. We hypothesized that hydrogen salts might have an effect on complex III, as it is the primary site of ROS production in mitochondria.

The present study used an *in vivo* I/R injury rat model to determine the effects of pharmacological postconditioning using hydrogen-rich saline on the activity of mitochondrial complex III.

Materials and Methods

Animals

The animal experiments in this study were approved by the

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***Corresponding author:** Yuanyuan Sun, Xiamen cardiovascular hospital, Xiamen University, Xiamen, 361004, China, Tel: +86-592-2292320; E-mail: guomingheart@163.com

Research Commission on Ethics of Xiamen Cardiovascular Hospital (Xiamen, China) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). A total of 96 Sprague-Dawley rats (male and female; weight, 200 gm-250 gm; age, 8 weeks) were obtained from Shandong University (Jinan, China). Rats were fed standard rat food (Beijing Vital River Laboratory Animal Technology Co., Ltd.) and kept in an on-site animal feeding facility at 22°C to 24°C, with a light-dark cycle of 12 h. There were eight rats in each cage, a total of 12 cages, and rats in each cage were randomly assigned to four experimental groups.

Rat heart model of acute myocardial ischemia and subsequent treatment

A rat model of acute myocardial infarction was established based on a previous study by Zhang et al. [15]. All rats were treated with Jinan. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (46 mg/kg). Following successful anesthesia, the neck skin and muscles of the rats were cut open for tracheal intubation and ventilator-assisted breathing using an hx-200 small animal ventilator with a tidal volume of 30 ml/kg, frequency of 50 times/min to 60 times/min and a respiratory ratio of 1:2. Subsequently, the chest was opened layer by layer, and a 5-0 suture was used to cross the deep part of the anterior descending branch between the lower margin of the left atrial ear and the pulmonary artery conus. The pressure of the NC Trek Coronary Dilatation Catheter (3 mm × 12 mm; Johnson & Johnson) was set at 1 atm and placed between the blood vessel and ligation line for ligation, and the pressure was rapidly adjusted to 12 atm for ischemia. After 45 min, the pressure pump was rapidly adjusted to 0 atm for reperfusion, and drug treatments were administered to the different groups, as described below.

Experimental protocols

The hydrogen-rich saline was prepared based on a previous study by Sun et al. [14,16,17]. NaOH (4 mol/l) was used to adjust the pH of the normal saline solution (0.9% NaCl) and hydrogen-rich saline to 7.4. Antimycin A was purchased from Beijing Zhongsheng Ruitai Technology Co., Ltd., and was prepared 1 h prior to injection.

Except for the sham operation group, the other rats underwent acute myocardial ischemia for 45 min followed by reperfusion (Figure 1). In all groups, the drugs were injected into the myocardial tissue around the infarct zone using a micro-injector. The rats were randomly divided into four groups: i) Sham group, in which the left coronary artery was opened, separated, and punctured without ligation. After 45-min of treatment, 60 μL normal saline was injected into the anterior wall of the heart; ii) I/R injury group, in which after 45 min of successful ischemia, the pressure pump was rapidly adjusted to 0 atm, and the balloon was removed. The ischemia site was immediately injected with a total of 60 μL normal saline at three points, and the reperfusion was maintained continuously; iii) Hydrogen-saline postconditioning (Hyd) group, in which after 45 min of successful ischemia, the pressure pump was rapidly adjusted to 0 atm, and the balloon was removed. The ischemic site was immediately injected with a total of 60 μL hydrogen-saturated saline at three points, and sustained reperfusion was maintained; and iv) hydrogen+antimycin A (Ant) group, in which after 45 min of successful ischemia, the pump was rapidly adjusted to 0 atm, and the balloon was removed. The ischemic site was injected with 60 μL of hydrogen-saturated saline and 60 μL of diluted antimycin A at three points, and reperfusion was maintained.

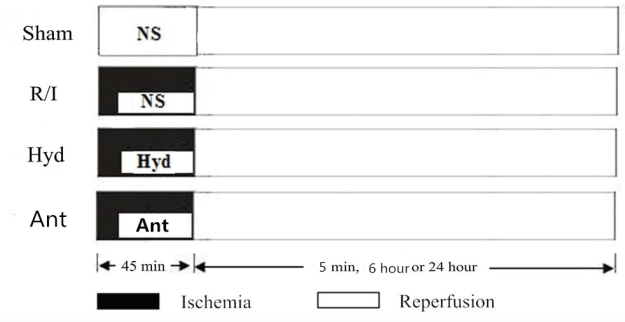

Figure 1: Timeline of the experimental protocols. The rats were received 45-min acute myocardial infarction, and then were injected with 0.9% normal saline (Sham and I/R groups), hydrogen-rich saline or hydrogen-rich saline with diluted antimycin A. White represents reperfusion; black represents ischemia. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning; Ant, hydrogen-rich saline and antimycin A postconditioning.

To collect the myocardial muscle and blood, the rats (8 rats in each group) were sacrificed at 5 min, 6 h, and 24 h after reperfusion. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (46 mg/kg). Following successful anesthesia, the rats were euthanized by spinal dislocation. The hearts were excised as described below (the anterior wall tissues of the left ventricles were kept at -80°C until further use) for the following measurements: i) ROS, the levels of Malondialdehyde (MDA) Hydroxylradical (·OH) and Superoxide Dismutase (SOD) activity were measured after 5 min of reperfusion; ii) the level of cytochrome b (Cyt-b) mRNA and the activity of complex III were measured to evaluate the function of complex III; iii) mitochondrial swelling and membrane potential were measured to indirectly evaluate the mitochondrial permeability transition pore (mPTP) opening; iv) the expression levels of Cyt-b and cytochrome c (Cyt-c) were determined after 6 h of reperfusion; v) the apoptotic index was measured after 24 h of reperfusion; and vi) the levels of myocardial serum markers and infarct size were measured 24 h after reperfusion by immediate staining.

Hemodynamic parameters

After successful anesthesia (intraperitoneal injection of urethane, 2 g/kg), the right carotid artery was cannulated using an arterial catheter and connected to a physiograph through a three-way stopcock. Heart rate and arterial pressure were monitored, and peak pressure differentials, +dp/dt max and -dp/dt max, were analyzed using physiography. The product of the heart rate and mean arterial pressure was the Rate-Pressure Product (RPP).

Levels of ROS in the myocardial tissue

The levels of MDA, ·OH, and SOD activity in the left ventricular myocardium were measured using a lipid peroxidation assay kit (Jian Cheng Company, Nanjing, China).

Reverse transcription-quantitative

analysis of Cyt-b in myocardial mitochondria: After reperfusion for 5 min, the ischemic myocardial tissue of each group was collected and total RNA was extracted using TRIzol® according to the manufacturer's instructions, and cDNA was synthesized by reverse transcription. A PCR instrument was used for amplification, and the primer sequences are listed in Table 1. The amplification conditions were as follows: 95°C for 20 s, 60°C for 35 s, and 72°C for 45 s for 32 cycles, followed by a final extension at 74°C for 3 min. According

Table 1: Oligonucleotide primer sets for real-time PCR.

Name	Sequence (5'-3')	Length	Tm	size
Cyt-bF	CCTATTATCTGCTATCCCTT	20	48.6	178
Cyt-b R	GTTATTTGATCCTGTTTCG	19	47.6	
β-actin F	GGAGATTACTGCCCTGGCTCCTAGC	25	60.1	155
β-actin R	GGCCGGACTCATCGTACTCCTGCTT	25	62	

to the method [18], the results were compared and analyzed using a fluorescence quantitative operation system, and the relative quantification of the target gene Cyt-b was calculated using the $2^{-\Delta\Delta Cq}$ method.

Detection of mPTP opening

The myocardial tissues from rats were washed with PBS three times, dried with filter paper, cut into small pieces, and isolated using the mitochondrial extraction and absorbance detection kit (Shanghai Genmed Pharmaceutical Technology Co., Ltd.) according to the manufacturer's instructions. The tissue homogenate was transferred to a centrifuge tube, centrifuged at 4°C for 10 min, and the supernatant was precipitated and transferred to new tubes. The precipitated mitochondria were obtained by centrifuging the supernatant at 4°C at 10,000 × g for 20 min. The mitochondria (200 μg protein) were diluted and treated with $CaCl_2$ (150 μmol/l) to induce the opening of mPTP. The mPTP opening assay was performed using an mPTP test medium containing 230 mM mannitol, 70 mM sucrose, and 3 mM HEPES (pH 7.4). The mitochondrial suspension was diluted to 1 μg/μl according to the mitochondrial concentration and placed on ice. A total of 3 ml mPTP test medium was placed into a cuvette as a blank control. The sample amount was calculated according to the protein concentration, supplemented to 3 ml with mPTP test medium to a final mitochondrial protein concentration of 0.5 ng/ml and mixed well to determine the optical density at 540 nm.

Complex III content was determined in stored mitochondrial suspensions using commercially available kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, and the optical density was measured at 550 nm.

Mitochondrial transmembrane potential analysis

Mitochondria (200 μg protein/group) were collected, and the JC-1 dye was proportionally diluted by adding 8 ml ultra-pure water for every 50 μl JC-1 (200X). Subsequently, 2 ml 5X JC -1 staining buffer was added and mixed to produce the JC-1 dyeing working fluid. The prepared JC-1 dyeing working fluid was diluted five times with 1X JC-1 dyeing buffer. A total of 20 μl resuspended mitochondria was placed in the micropore, and 180 μl JC-1 dyeing working fluid was added. An excitation wavelength of 485 nm, an emission wavelength of 525 nm, an excitation wavelength of 525 nm, and an emission wavelength of 590 nm were used to determine the membrane potential using a fluorescence microplate reader.

Activity of the mitochondrial complex III

Mitochondria were extracted from myocardial tissue by differential centrifugation. The ice bath homogenate of myocardial tissue was centrifuged at 1300 g at 4°C for 10 min and repeated three times. The supernatant was then centrifuged at 17000 g for 15 min and repeated three times. The supernatant was discarded and the precipitate was mitochondria. The activity levels of mitochondrial complex III were measured using a Complex III Activity Test kit (Jian Cheng Company, Nanjing, China).

Western blot analysis of Cyt-b in mitochondria and Cyt-c in the cytoplasm

Mitochondria were isolated by differential centrifugation for Cyt-b analysis, and the cytoplasm was extracted for Cyt-c analysis. Equal amounts of cell lysate proteins (30 μg) were extracted using RIPA protein extraction buffer with a protease inhibitor, separated by 10% to 14% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked and incubated with primary antibodies (1:400; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following washing, the membranes were incubated with a secondary antibody (1:5,000; Amersham; Cytiva) for 1 h at room temperature. The antigen and antibody complexes were visualized and analyzed by the ECL method using Image-Pro Plus image analysis software (version 4.1; Media Cybernetics, Inc.). The relative expression levels of the target proteins were calculated as the ratios of target protein IOD value to COX IV or β-actin IOD value using rabbit anti-rat COX IV or β-actin (1:200) monoclonal antibodies as the control.

Detection of apoptotic cells

The apoptotic index was determined as described previously [16]. The rat hearts were collected 24-h post-reperfusion, and the ischemic area of the myocardial tissue was dissected and placed in neutral formaldehyde and fixed for 24 h prior to paraffin embedding. Apoptosis was detected *in situ* by dUTP notch-end labeling, mediated by terminal deoxynucleotide transferase. Images were captured under a microscope, and five high-power fields (x400 magnification) were selected for each section. The number of apoptotic cells per 100 cells was calculated and used as the apoptotic index.

Serum markers of myocardial muscle injury

Blood was collected from the rats after 24-h of reperfusion, and the sample was immediately centrifuged at 1,409 × g for 10 min. The serum levels of troponin T (TnT) and the MB isoenzyme of creatine kinase (CK-MB) were analyzed using an automatic clinical chemistry analyzer (BPC Biosed Srl).

Measurement of infarct size

The infarct size was determined as previously described by Kerendi et al. [16]. Area analysis was performed using Image-Pro Plus software to determine the ischemic cardiac risk area (AAR), necrotic area (AN), and left ventricular area (LV). The ischemic area was represented as the ratio of AAR to LV area, and the infarct area was represented by the ratio of AN to AAR.

Statistical analysis

Data are presented as the mean ± S. Data was analyzed using SPSS (version 13.0; IBM Corp.). Differences were evaluated using Student's t-test (unpaired) or one-way analysis of variance (ANOVA). A two-tailed $P<0.05$, was considered to indicate a statistically significant difference.

Results

Hemodynamic parameters

Recordings of the hemodynamic parameters heart rate and Mean Arterial Pressure (MAP) demonstrated that heart contractility was not significantly different among the experimental groups (Table 2). In contrast, there were changes in +dp/dt max and -dp/dt max. The values of +dp/dt max and -dp/dt max in the I/R group were lower than those in the sham group ($P<0.05$). The values of +dp/dt (3597.67 ± 218.18 *vs.* 2840.24 ± 304.21) max and -dp/dt max (2819.33 ± 336.12 *vs.*1825.01 ± 337.76 mmHg/s) in the Hyd group were higher compared

with those in the I/R group (P<0.05). Compared with those in the Hyd group, these measurements in the Ant group were significantly lower (P<0.05); however, they were significantly higher in the Ant group than in the I/R group (P<0.05).

MDA, OH, and SOD levels

MDA, ·OH, and SOD activity levels were used as indirect measures of ROS to determine the effects of each treatment on the production of ROS (Table 3). The results demonstrated that the MDA content and ·OH activity were higher (P<0.05), and SOD activity was lower in the I/R group than in the sham group (P<0.05). The MDA content (2.97 ± 0.48 *vs.* 4.43 ± 0.43 nmol/mgpro) and ·OH activity (18.11 ± 2.35 *vs.* 9.55 ± 1.16 U/mgpro) were significantly lower in the Hyd group compared with those in the I/R group (P<0.05), whereas SOD activity was significantly higher in the Hyd group compared with that in the I/R group (15.22 ± 0.83 *vs.* 9.55 ± 1.16 U/mgpro P<0.05). In the three free radical assays, the results in the Ant group were between those in the I/R and Hyd groups, and all of them exhibited significant differences (P<0.05).

Effects of hydrogen-rich saline on Cyt-b expression in complex III

The mRNA and protein levels of Cyt-b, which is an important component of complex III, were quantified in myocardial tissues from all groups (Figure 2). The levels of Cyt-b in the Hyd group were significantly higher compared with those in the I/R group (0.59 ± 0.06 *vs.* 0.38 ± 0.08; P<0.05). The Cyt-b expression levels in the Ant group were significantly higher compared with those in the I/R group (0.60 ± 0.08 *vs.* 0.38 ± 0.08; P<0.05), and no significant difference was observed between the Ant and Hyd groups (0.60 ± 0.08 *vs.* 0.59 ± 0.06; P<0.05).

Respiratory chain complex III activity in the ischemic myocardial tissue

Complex III activity levels were measured to elucidate the effects of each treatment on mitochondrial function (Table 3). The levels of complex III activity in the I/R group were lower than those in the sham group (P<0.05). Complex III activity levels in the Hyd group were significantly higher than those in the I/R group (P<0.05). Complex III activity levels in the Ant group were significantly lower than those in the Hyd group (P<0.05) and higher than those in the I/R group, although the difference was not significant (P>0.05).

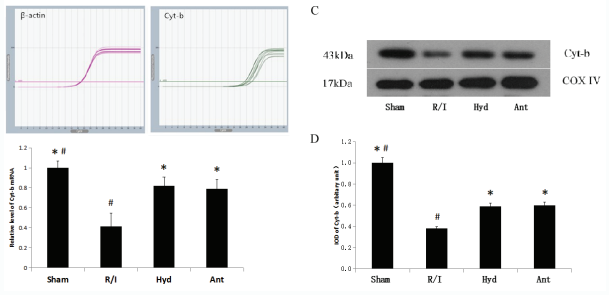

Figure 2: Cyt-b mRNA and protein expression in the cytoplasm. (A) RT-PCR analysis of Cyt-b in the total RNA extracted from myocardial tissues. (B) RT-quantitative PCR analysis of Cyt-b in the total RNA extracted from myocardial tissues. (C) Western blot analysis of the protein expression levels of Cyt-b. (D) Densitometry of western blot bands normalized to COX IV. The gels were run under the same experimental conditions. Cyt-b protein levels in the mitochondria of the I/R group were lower compared with those in the Sham group, and those in the Hyd group were significantly higher compared with those in the I/R group. The levels of Cyt-b in the Ant group were significantly higher compared with those in the I/R group, and there was no significant difference between the Ant and Hyd groups. n=8 rats/group. *P<0.05 *vs.* I/R; #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group; cytochrome b (Cyt-b), reverse transcription (RT). We cut out the periphery of the original strip for the sake of aesthetic image.

Mitochondrial absorbance and membrane potential

Mitochondrial absorbance and membrane potential were determined in different experimental groups (Figure 3). The mitochondrial absorbance and membrane potential in the I/R group were lower than those in the sham group (P<0.05). In the Hyd group, the mitochondrial absorbance and membrane potential were significantly higher than those in the I/R group after 5-min of reperfusion (P<0.05). The two measures in Ant the group were higher compared with those in the I/R group and lower compared with those in the Hyd group (Ant *vs.* I/R: mPTP absorbance, 0.63 ± 0.12 *vs.* 0.45 ± 0.06; P<0.05; membrane potential, 0.88 ± 0.09 *vs.* 0.65 ± 0.11; P<0.05; Ant *vs.* Hyd, mPTP absorbance, 0.63 ± 0.12 *vs.* 0.78 ± 0.06; P<0.05; membrane potential, 0.88 ± 0.09 *vs.* 0.99 ± 0.09; P<0.05).

Effects of hydrogen-rich saline on Cyt-c expression levels in the cytoplasm

To determine the potential involvement of this pathway in

Table 2: Hemodynamic parameters of each group after AMI and reperfusion (mean ± SD) (n=8).

Group	HR (bpm)	MAP (mmHg)	+dP/dt (mmHg/s)	-dP/dt (mmHg/s)
Sham	361.32 ± 21.26	100.21 ± 9.17	3862.88 ± 260.62*#	3303.50 ± 186.12*#
I/R	355.61 ± 20.23	99.32 ± 11.41	2840.24 ± 304.21#	1825.01 ± 337.76#
Hyd	363.27 ± 26.15	99.81 ± 9.76	3597.67 ± 218.18*	2819.33 ± 336.12*
Ant	364.18 ± 31.29	104.01 ± 10.72	3219.26 ± 268.71*#	2423.18 ± 256.56*#

HR: Heart Rate; bpm: Beats per Minute; MAP: Mean Arterial Pressure

*P< 0.05 *vs.* R/I, #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group.

Table 3: The level of MDA, SOD, •OH, complex III activity and serum markers of cardiac damage (mean ± SD) (n=8).

Group	MDA (nmol/mgpro)	SOD (U/mgpro)	·OH (U/mgprot)	Complex III activity (U/mgprot)	TnT (U/L)	CK-MB (U/L)
Sham	2.45 ± 0.42*#	17.17 ± 0.94*#	11.27 ± 1.16*#	5.35 ± 1.04*#	176.18 ± 31.28*#	78.96 ± 34.91*#
I/R	4.43 ± 0.43#	9.55 ± 1.16#	27.27 ± 3.53#	1.43 ± 0.69#	1290.36 ± 211.43#	989.43 ± 173.26#
Hyd	2.97 ± 0.48*	15.22 ± 0.83*	18.11 ± 2.35*	3.52 ± 0.31*	756.21 ± 289.15*	489.21 ± 98.28*#
Ant	3.51 ± 0.27*#	12.15 ± 1.05*#	22.35 ± 4.70*#	1.45 ± 0.43	987.21 ± 233.62*#	678.21 ± 86.15*#

MDA: Malondialdehyde; SOD: Superoxide Dismutase; CK-MB: MB Isoenzyme of Creatine Kinase; *P< 0.05 *vs.* R/I, #P<0.05 *vs.* Hyd.I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group.

pharmacological postconditioning, Cyt-c protein expression levels were determined by western blotting (Figure 4). Following 24-h reperfusion, the protein levels of Cyt-c in the cytoplasm of the I/R group were higher compared with those in the Sham group (P<0.05), and the levels in the Hyd group were significantly lower compared with those in the I/R group (1.90 ± 0.13 *vs.* 3.81 ± 0.12; P<0.05). Antimycin A treatment significantly lowered the Cyt-c protein levels compared with those in the I/R group (2.94 ± 0.21 *vs.* 3.81 ± 0.12; P<0.05); however, the expression levels of Cyt-c in the Ant group were higher than those in the Hyd group (2.94 ± 0.21 *vs.* 1.90 ± 0.13; P<0.05).

Cardiomyocyte apoptosis

The I/R model induced a significant increase in the apoptotic index of cardiomyocytes compared with that in the sham group (P<0.05), as determined by the TUNEL assay (Figure 5). Whereas that in the Hyd group was significantly lower compared with that in the I/R group (13.67 ± 3.77 *vs.* 30.57 ± 6.19%; P<0.05). The apoptotic index of the Ant group was significantly lower than that of the I/R group (20.95 ± 6.79 *vs.* 30.57% ± 6.19%; P<0.05), but significantly higher than that in the Hyd group (20.95 ± 6.79 *vs.* 13.67 ± 3.77%; P<0.05).

Serum markers of cardiac damage

The I/R model induced significant increases in the blood levels of CK-MB and TnT in the I/R group compared with those in the sham group (P<0.05) (Table 3). All treatment groups exhibited significantly decreased levels of CK-MB and TnT compared to those in the I/R group (all P<0.05). The serum levels of CK-MB and TnT in the Ant group were significantly higher than those in the Hyd group (P<0.05).

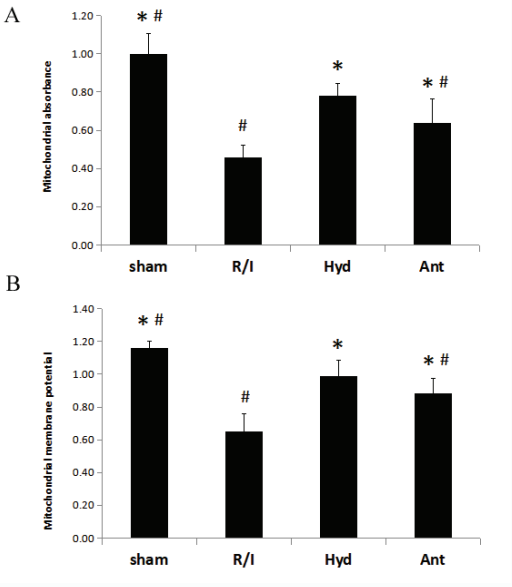

Figure 3: Mitochondrial absorbance and membrane potential reflecting mitochondrial permeability transition pore opening. (A) Mitochondrial absorbance. (B) Mitochondrial membrane potential. The mitochondrial absorbance and membrane potential in the I/R group were lower compared with those in the Sham group. The mitochondrial absorbance and membrane potentialin the Hyd group were significantly higher compared with those in the I/R group. The two measures in the Ant group were higher compared with those in the I/R group and lower compared with those in the Hyd group. n=8 rats/group. *P<0.05 *vs.* I/R; #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group.

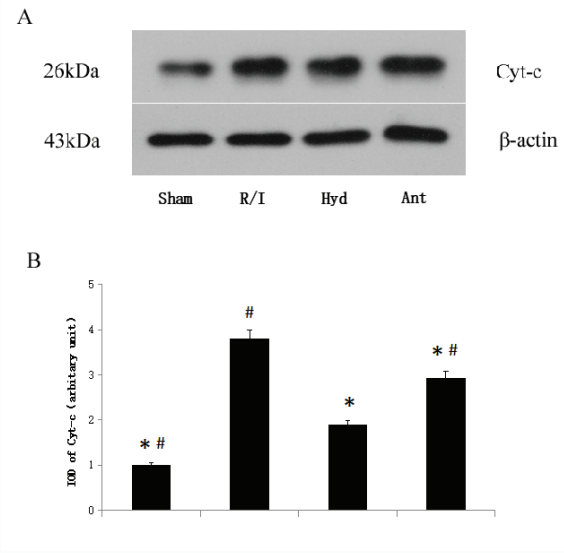

Figure 4: Cyt-c protein expression in the cytoplasm. (A) Western blot analysis of the protein expression levels of Cyt-c. (B) Densitometry of the western blot bands normalized to β-actin. The gels were run under the same experimental conditions. The protein levels of Cyt-c in the cytoplasm of the I/R group were higher compared with those in the Sham group, and the levels in the Hyd group were significantly lower compared with those in the I/R group. The Cyt-c protein expression levels the in Ant groups were significantly lower compared with those in the I/R group, but higher compared with those in the Hyd group. n=8 rats/group. *P<0.05 *vs.* I/R; #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group; Cyt-c, cytochrome c. We cut out the periphery of the original strip for the sake of aesthetic image.

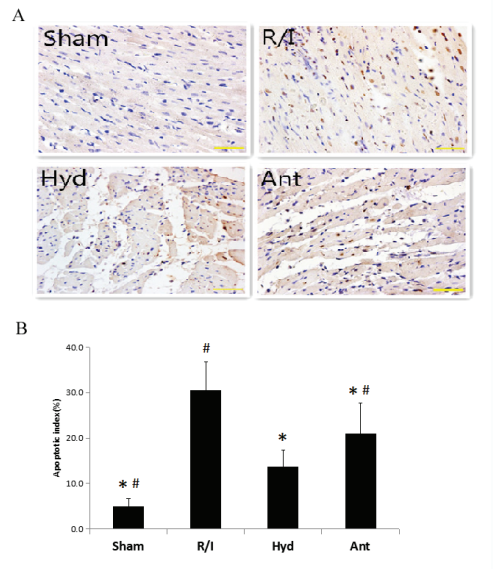

Figure 5: Apoptosis detection using the TUNEL assay. (A) Detection of apoptotic cells using TUNEL staining. Normal cells were stained blue, whereas apoptotic cells were stained brown. (B) The myocardial apoptotic index of the I/R group was higher compared with that of the Sham group, whereas that of the Hyd group was significantly lower compared with that of the I/R group. The apoptotic index of the Ant group was significantly lower compared with that of the I/R group, but significantly higher compared with that of the Hyd group. n=8 rats/group. *P<0.05 *vs.* I/R; #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group.

Infarct size

The infarct size was determined by histological analysis (Figure 6). The AAR zone was stained red and white, whereas the AN zone was stained white. The size of the ischemic area, expressed as a percentage of the area of the left ventricle (AAR/LV), was similar among the groups (50.01% to 53.08%). The infarct size, expressed as a percentage of the area at risk (AN/AAR), was significantly lower in the Hyd (10.01 ± 1.2%) and Ant (14.21 ± 2.6%) groups compared with that in the I/R group (18.98 ± 3.2%) (both P<0.05). The infarct size in the Ant group was higher than that in the Hyd group (10.01 ± 1.2 *vs.* 14.21 ± 2.6%; P<0.05).

Discussion

Several studies have focused on the pathophysiological and regulatory mechanisms underlying I/R injury, and various strategies have been devised to prevent its occurrence [19-23]. These strategies include mechanical and pharmacological postconditioning strategies. However, mechanical postconditioning can lead to deleterious consequences, such as distal embolism. In contrast, pharmacological postconditioning has demonstrated promising results [24-28]. Hydrogen was once regarded as a physiological inert gas without significant effects on biological systems, and in 2007, Ohsawa et al. [13] demonstrated that hydrogen significantly reduced cerebral infarction volume 24 h after cerebral I/R. Their study prompted investigations into the potential therapeutic actions of hydrogen postconditioning. The strong permeability across biological barriers, the ability to enter various tissues in the body, and excellent safety profiles without significant side effects make hydrogen an excellent candidate therapeutic medical gas [29]. The results of the present study demonstrated that hydrogen saline postconditioning played a role in myocardial protection in a rat model of I/R injury, and that the underlying mechanism may involve the selective antagonism of hydrogen against ROS.

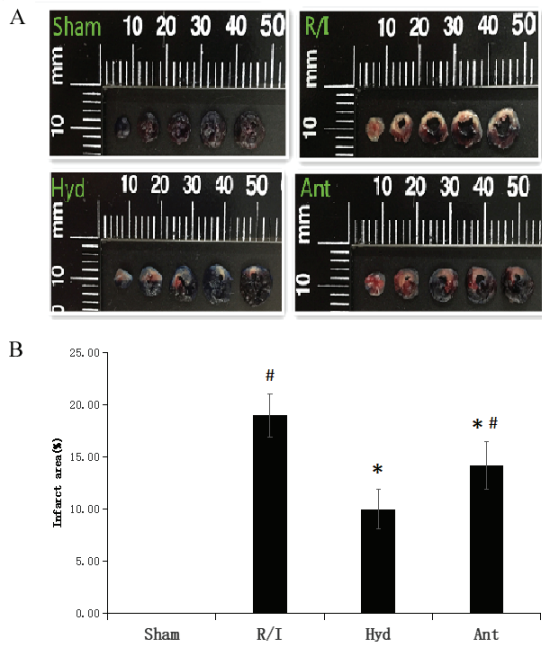

Figure 6: Quantification of the infarct area size. (A) Ischemic and infarct areas in all groups; blue indicates a non-ischemic zone (dyed by Evans blue), red indicates an area of ischemia without infarction, and white indicates an infarct zone (dyed by triphenyltetrazolium chloride). (B) Infarct sizes for all groups. The area of ischemia was similar among all groups; the infarct size in the Hyd and Ant groups was significantly lower compared with that in the I/R group. The infarct size in the Ant group was higher compared with that in the Hyd group. *P<0.05 *vs.* I/R; #P<0.05 *vs.* Hyd. I/R, ischemia-reperfusion injury; Hyd, hydrogen-rich saline postconditioning group; Ant, hydrogen-rich saline and antimycin A postconditioning group.

Mitochondria are one of the most important organelles in cardiomyocytes, accounting for 45% of the total cell volume. Following I/R, oxygen free radicals are mainly derived from mitochondria, in which the respiratory chain complex III is the main site for ROS generation [30]. Considering the important role of ROS in I/R injury, we hypothesized that changes in the levels and types of ROS in the postconditioning process may be associated with changes in the function and structure of the respiratory chain complex III. In the present study, the Hyd group exhibited significantly improved activity of the respiratory chain complex III compared with that in the I/R group, as well as reduced generation of free radicals, especially ·OH, which causes tissue damage if released in large amounts.

Cyt-b, which is an important component of complex III, transmits electrons from coenzyme Q to ferrothionein. In the present study, the protein expression levels of Cyt-b in the Hyd group were significantly higher than those in the I/R group 5 min and 6 h after reperfusion, suggesting that post-conditioning with hydrogen-rich saline protected the structure of complex III. Antimycin A can inhibit one of the two pathways mediating cyclic electron flow and has been widely used to characterize the mitochondrial respiratory chain. Antimycin A has been used since 1963 for the characterization of cyclic electron flow when ferredoxin was identified as the electron donor in the pathway. Although antimycin A inhibits the function of complex III, it does not affect the expression of Cyt-b, as demonstrated by the results of the present study. This suggests that the activity of complex III is not completely dependent on the levels of Cyt-b expression, possibly because antimycin A does not affect the structure of complex III despite blocking the electron transition in it [31].

The mPTP is a protein complex located in the mitochondrial membrane and is considered the final effector of the cell protection pathway [32]. The opening of the mPTP leads to increased osmotic pressure in the mitochondrial membrane space, followed by mitochondrial swelling and rupture of the outer membrane. Certain pro-apoptotic factors, such as Cyt-c, are released from the membrane space to the cytoplasm, triggering apoptosis. If a large number of mPTPs are opened within a short period of time, the aforementioned changes will lead to alterations in energy generation and cell death by apoptosis and necrosis.

Recently, molecular hydrogen has been tested in preclinical and clinical studies on various diseases associated with oxidative and inflammatory stress, such as radiation-induced heart disease, I/R injury, and myocardial and brain infarction. Hydrogen favorably modulates signal transduction and gene expression, resulting in the suppression of proinflammatory cytokines, excess ROS production, and activation of the Nrf2 antioxidant transcription factor. A previous study has demonstrated that hydrogen-rich saline alleviates the inflammatory response and apoptosis induced by I/R and contributes to the expression of proteins associated with autophagy, which may act via Pink1/Parkin-mediated mitophagy.

Ischemic preconditioning and ischemic postconditioning (I-PostC) induce endogenous protective mechanisms that attenuate

reperfusion injury. However, repeated transient I/R cycles used in preconditioning and postconditioning may cause injury, which is a drawback that limits its clinical use. Recently, attempts have been made to substitute drug administration for transient I/R cycles used in I-PostC. The resulting Pharmacological Postconditioning (PPC) has exhibited potential in both animal studies and clinical practice. Commonly used drugs in PPC include anesthetics, adenosine, levosimendan, and statins. PPC avoids the potential injury induced by I-PostC, is easier to perform, and therefore has improved clinical potential compared with that of I-PostC.

The results of the present study demonstrated that hydrogen-rich saline treatment effectively inhibited the opening of mPTP, and the protein expression levels of Cyt-c in the cytoplasm were significantly lower in the Hyd group than in the I/R group, whereas the apoptotic index was significantly reduced. However, the protective effect of hydrogen-rich saline was significantly weakened by a complex III blocker. Although the expression levels of Cyt-c in the Ant group were similar to those in the I/R group, mPTP opening and apoptosis levels in the Ant group were significantly reduced. These results suggest that the myocardial protective effect of hydrogen may be largely attributed to the activity of complex III.

The aforementioned results indicate that complex III may play an important role in pharmacological postconditioning with hydrogen-rich saline. However, the protective effects induced by hydrogen-rich saline were not completely abolished by the pharmacological inhibition of complex III, suggesting the involvement of other regulatory pathways. In addition, the subtypes of Cyt-b were not quantified in the present study, and future studies should investigate the relative contributions of these subtypes. Another limitation of the present study was the lack of assessment of the effects of antimycin A on the concentrations of SOD and ·OH.

Conclusion

In conclusion, hydrogen-rich saline postconditioning increased the levels of complex III activity and mitochondrial transmembrane potential, and alleviated myocardial apoptosis in rats following I/R injury.

Declarations

Ethics approval and consent to participate

The experimental protocol was established according to the ethical guidelines set forth in the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, NIH) and was approved by the Research Commission on Ethics of Xiamen Cardiovascular Hospital.

Availability of Data and Materials

The datasets used or analyzed in the current study are available from additional supporting files.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

GZ and YS analyzed and interpreted the experimental data regarding the mitochondria and cellular protective pathways. LD, GS, and LC performed the animal experiments and molecular biological detection; GS, BW, and TYwere responsible for the consultation of experimental methods. YS and GZ were the major contributors to writing the manuscript. All authors have read and approved the final manuscript.

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